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Chromatin remodeling is one of the most intriguing features of spermiogenesis, during which nuclei undergo drastic morphological changes leading to extensive nuclear compaction. It is essential for sperm function and proper behavior of paternal chromosomes in the zygote. In a highly coordinated manner, paternal histones are replaced with protamine-like proteins leading to a high degree of nuclear condensation. Key players of chromatin remodeling in *Drosophila* include histones, histone variants, transition protein-like proteins and protamine-like proteins. In addition, several paternal-effect genes required for paternal chromosome transmission in the early embryo may also play a role in sperm chromatin remodeling. Here, we characterize a new male-specific mutation in the fruit fly, *mcl(3)Z2566* that causes fourth and sex chromosome loss. Previous experiments pointed to *CG5538* as the candidate gene. To verify that *mcl(3)Z2566* corresponds to *GC5538*, we showed that a transgene expressing EGFP-labeled wildtype *CG5538* rescued the mutant. We also inhibited *CG5538* expression with siRNA and generated a missense mutation in *CG5538*, both which recapitulated the chromosome loss phenotype. We found that *CG553-EGFP* was nuclear localized and expressed at the canoe stage of spermiogenesis. We conclude that *CG5538* is a novel paternal-effect gene that functions during the histone-to-protamine transition and is important for chromosome behavior subsequent to fertilization.

*MALE CHROMOSOME LOSS(3)Z2566 IS REQUIRED FOR*  
*PATERNAL CHROMOSOME TRANSMISSION IN*  
*DROSOPHILA MELANOGASTER*

by

Andrea Maria Binder

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Approved by

John Tomkiel Dean  
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To my wife Penelope,  
our children Sean and Amelie and to my family.  
I matter because of you.

## APPROVAL PAGE

This thesis, written by Andrea Maria Binder, has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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## CHAPTER I

### INTRODUCTION

Developmentally regulated changes in the structure and nuclear organization of chromatin are widely recognized as critical for regulating gene expression. Perhaps the most intriguing and dramatic of these changes occur in spermiogenesis, during which the haploid sperm chromatin is structurally reorganized resulting in drastic morphological changes to the sperm nucleus. These changes affect not only gene expression, but also are necessary for sperm function and for the proper behavior of chromosomes once they are delivered to the zygote. During this highly coordinated process the chromatin is opened up and paternal histones are replaced with protamine-like proteins (ProtA and ProtB) in the mature sperm allowing for a higher degree of condensation. The reorganization of chromatin with protamines, resulting in morphological changes to the sperm head, is a feature conserved between fruit flies and mammals (Rathke, Baarends et al. 2007). The similarity of this histone-to-protamine transition in fruit flies and mammals makes *Drosophila* an excellent model organism for in-depth studies of spermiogenesis and to characterize paternal-effect genes required for post-fertilization in the early embryo.

## **Chromatin Remodeling during Spermiogenesis**

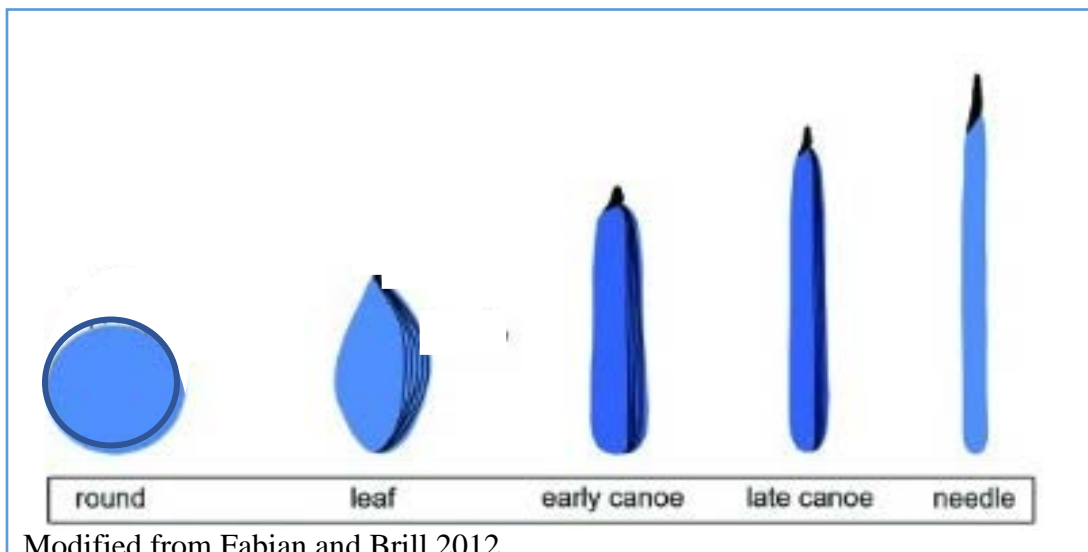
In *Drosophila*, sperm production begins with the division of germline stem cells in the apical tip of the testis to produce spermatogonia, which undergo four rounds of mitoses to produce sixteen primary spermatocytes. Spermatocytes then go through the two divisions of meiosis to produce spermatids, which differentiate and mature during spermiogenesis. The first stage of spermiogenesis is called the onion stage, which consists of a syncytial cyst of 64 haploid spermatids (Tokuyasu, Peacock et al. 1972). At this stage the mitochondria coalesce into layered, spherical structures called nebenkerns, resembling the layers of an onion. It is also at this stage that the spermatids begin to undergo a series of chromatin remodeling steps combined with drastic morphological changes.

A number of proteins have been identified in flies that have a role in this chromatin remodeling. These include the histones themselves, histone variants, transition-protein-like proteins that displace nucleosomes during the early stage of remodeling, and protamine-like proteins that ultimately substitute for the majority of the histones in the mature sperm. In the nucleus, DNA wraps around octamers of core histones comprised of H2A, H2B, H3 and H4. The octamers are connected by linker DNA and are stabilized in this bead structure by H1, a linker histone, sitting outside of the core complex. The core histones are involved in genome packaging and gene regulation is controlled epigenetically via modifications such as acetylation, methylation

and ubiquitination that open up or condense chromatin (Raychaudhuri, Dubruille et al. 2012).

The post-meiotic morphogenesis of sperm in *Drosophila* has been categorized into the following stages: leaf, early canoe, late canoe and needle (Fabian and Brill 2012). By the leaf stage, the previously round spermatid nucleus has transformed to a leaf-like shape in which the position of the future acrosome is clearly defined. At the early canoe stage the round spermatids begin to elongate into an oblong shape as chromatin is opened up for remodeling. Chromatin reorganization and subsequent condensation causes spermatid nuclei to become thinner in the late canoe stage. At the needle stage, full chromatin condensation is achieved and the spermatids individualize and are considered mature (Fabian and Brill 2012), (Figure 1).

**Figure 1. Stages of Spermiogenesis in *Drosophila melanogaster*.**



During these chromatin remodeling steps, indirect immunofluorescence staining with modification-specific antibodies revealed that a number of epigenetic modifications occur on the core histones that precede their removal. H2A and H2B are marked by ubiquitination and four lysine residues in the histone tails of H4 (K5, K8, K12, K16) are hyperacetylated (Rathke, Baarends et al. 2007). The hyperacetylation, often combined with H3K4 mono-, di-, and trimethylation, is thought to open up the chromatin for further changes. Since this has been observed prior to histone removal it is assumed that it grants easier access of a transcriptional control complex comprised of testis-specific TATA-box-binding-protein-associated factors (tTAFs) to the histones and aids in the accessibility of transition protein-like proteins Tpl94D, tHMG-1 and tHMG-2 (Rathke, Baarends et al. 2007). The spherical nuclei of the onion stage spermatids begin to elongate as the core histones are removed and the chromatin begins to condense. Condensation of the chromatin is associated with deacetylation and mono-, di-, trimethylation of known epigenetic repressive marks including H3K9 and H3K27 (Rathke, Baarends et al. 2007).

As the sperm progress into the early canoe stage, transition-protein-like proteins including Tpl94D, tHMG-1 and tHMG-2 are expressed with levels peaking at the histone-to-protamine transition (Gartner, Rothenbusch et al. 2015). Transition proteins (TPs) are small basic proteins expressed in sperm of mammals and contain a DNA-binding HMG-box domain (Rathke, Baarends et al. 2007) which can bind, unwind and bend DNA thus changing its shape (Stros 2010). During the histone-to-protamine transition, TPs replace histones in the chromatin and are later replaced by protamines in

the maturing sperm. Transition proteins have not yet been identified in *Drosophila*, however Rathke (Rathke, Baarends et al. 2007) and Gartner (Gartner, Rothenbusch et al. 2015) were able to characterize functional homologs in *Drosophila*, which are called transition-protein-like proteins, namely Tpl94D (Rathke, Baarends et al. 2007), tHMG-1 and tHMG-2 (Gartner, Rothenbusch et al. 2015). tHMG-1 and tHMG-2 expression starts early in the spermatocytes with levels peaking at the late canoe stage, while Tpl94D expression is not observed until the early canoe stage and also peaks at late canoe stage (Gartner, Rothenbusch et al. 2015). The exact functions of tHMG1 and tHMG2 are yet to be determined but it is known that they form a heterodimer with each other and co-localize along with Tpl94D to chromatin during the late canoe stage (Gartner, Rothenbusch et al. 2015).

During the late canoe stage, the transition-protein-like proteins are removed and protamines are loaded onto the sperm chromatin. The mature needle stage sperm DNA is packaged largely by protamines, but a small but biologically significant fraction of histones remain. Notably, CID, a centromere-specific H3 variant, is incorporated into the centromere region to replace H3 and is retained in mature sperm. It acts as an epigenetic mark for centromere assembly. (Raychaudhuri, Dubruille et al. 2012). CID is essential for centromere function of the paternal chromosomes in early embryogenesis and its absence results in paternal chromosome loss. During early embryogenesis, paternal chromosomes deficient of CID are not able to acquire maternal CID resulting in centromere defects (Raychaudhuri, Dubruille et al. 2012).

In addition, Mst77F, a protein similar to the linker histone H1, is also found in mature sperm (Rathke, Barckmann et al. 2010). Mst77F, which is specifically expressed post-meiotically, has a strong positive charge and interacts with DNA via nonspecific electrostatic interactions with the sugar-phosphate backbone, causing major condensation and tight packaging of the DNA (Kost, Kaiser et al. 2015). Additionally, Mst77F coordinates microtubules during nuclear shaping when sperm heads change from spherical to needle shaped with condensed chromatin (Rathke, Barckmann et al. 2010). Mutation of Mst77F leads to unstable microtubules and to defects in sperm head formation (Rathke, Barckmann et al. 2010).

Protamines are small, testis-specific sperm nuclear proteins found in *Drosophila* and many mammalian species (Barckmann, Chen et al. 2013). They are involved in the packaging of the sperm DNA into the sperm head as they replace the larger histones. This allows for a compact sperm head structure conferring an advantage in sperm mobility. While the chromatin in the mature sperm of mammalian organisms is packaged by two protamines (Protamine 1 and 2) enriched with arginine residues, *Drosophila* sperm chromatin is loaded with the protamine-like proteins ProtA (Protamine A) encoded by *Mst35A* and ProtB (Protamine B) encoded by *Mst35B*. ProtA and ProtB are nearly identical and are conserved among other *Drosophila* species (Tirmarche, Kimura et al. 2014) and are rich in cysteine residues (Rathke, Barckmann et al. 2010). Regulation of expression of the protamines, as well as MstF77, occurs post-transcriptionally. *Mst35A*, *Mst35B* and *Mst77F* are transcribed in the primary spermatocytes, as are the majority of genes involved in spermiogenesis, however their mRNAs are translationally repressed by



tTAFs until the early canoe stage (Barckmann, Chen et al. 2013). The tTAFs contain bromodomains that recognize and bind to the lysine-rich tails of the core histones. Five tTAFs have been identified in *Drosophila*: Cannonball (Can; dTAF5 homolog), No hitter (Nht; dTAF4 homolog), Meiotic arrest (Mia; dTAF6 homolog), Spermatocyte arrest (Sa; dTAF8 homolog), and Ryan express (Rye; dTAF12 homolog). These tTAFs have been proposed to act as a complex during *Drosophila* spermiogenesis (Hiller, Chen et al. 2004). Mutations in any of these genes prevents the spermatocytes from entering meiosis and thus disrupt spermiogenesis (Barckmann, Chen et al. 2013). Once their messages are translated at the canoe stage, ProtA, ProtB and Mst77F are incorporated into the chromatin and remain there until fertilization and the subsequent decondensation of the sperm pronucleus in the early embryo (Tirmarche, Kimura et al. 2014).

## **Fertilization**

At fertilization in *Drosophila* the sperm enters the egg with the plasma membrane intact (Wilson, Fitch et al. 2006). Embryogenesis follows an exact developmental program in which key components perform their tasks in a highly coordinated and chronological order. Once inside the egg regulatory factors present in the egg cytoplasm quickly activate the compacted sperm nucleus. As a result the sperm plasma membrane breaks down and sperm-specific membrane proteins are exchanged with maternal proteins. Following sperm nuclear decondensation and conversion into a male pronucleus directed by maternally loaded regulatory factors such as the sésame protein, sperm-specific protamines are replaced with maternal histones. Sésame is directly involved in

sperm nuclear decondensation and in maternal histone incorporation into the paternal chromatin (Loppin, Berger et al. 2001). Mutations of *sésame* have shown to result in sperm pronuclear development arrest and as a consequence to halted embryogenesis (Loppin, Berger et al. 2001).

After sperm decondensation, sperm and egg pronuclei fuse to form the early zygote, which then enters the first mitotic division. Although maternal-effect genes regulate much of early embryogenesis, a small number of paternal genes are also required during these early stages (Wakimoto, Lindsley et al. 2004). Thus, the sperm not only delivers the paternal complement of chromosomes, but also is involved in essential early developmental events.

### **Paternal-effect Genes**

Studies of paternal effect mutations have revealed that several aspects of chromosome transmission in the early embryo are paternally controlled. Mutation of the *paternal chromosome loss (pal)* gene in males causes the loss of paternally-derived chromosomes in developing embryos during the early mitotic divisions. The effect of the mutation is male-specific and the protein is likely only required in sperm for post-fertilization behavior of chromosomes (Baker 1975).

*Horka* is a dominant paternal effect mutation affecting all chromosomes except the Y chromosome (Szalontai, Gaspar et al. 2009). Horka protein is needed for proper centromere function and segregation during the metaphase to anaphase transition, and its

mutation causes chromosome instability and subsequent chromosome loss in the embryo, leading to the formation of diplo//haplo mosaics (Szabad, Mathe et al. 1995).

The mutation *male sterile K81* (*ms(3)K81*) has a similar but more extreme phenotype of complete loss of paternal chromosomes at the first embryonic division (Yasuda, Schubiger et al. 1995). The K81 protein is needed to protect the telomeres on paternal chromosomes in the embryo during early mitosis (Dubruille, Orsi et al. 2010) (Gao, Cheng et al. 2011). If *ms(3)K81* is absent the telomeres of sister chromatids of paternally derived chromosomes fuse. As a result anaphase bridges form and the entire paternal complement of chromosomes is lost at the first division, resulting in non-viable haploid embryos (Gao, Cheng et al. 2011). During sperm maturation K81 replaces an analogous mitotic protein called Hiphop in order to package chromatin into the small sperm head. After fertilization, chromatin is remodeled again and Hiphop replaces K81 in the embryo (Gao, Cheng et al. 2011).

A fourth paternal-effect gene, *loser*, has been identified that also affects paternal chromosome transmission (Wakimoto, Lindsley et al. 2004), but has not yet been fully characterized.

Here, we characterize a new male-specific mutation in the fruit fly, *mcl(3)Z2566*. This mutation was originally isolated in screen for mutations that caused 4<sup>th</sup> chromosome loss in males (Wakimoto, Lindsley et al. 2004), and was previously mapped by recombination and deletion mapping to salivary chromosome band 87B11 (C. Davis, J. Chmielewski and J. Tomkiel Dean, unpublished). Chromosome loss of both the sex and

fourth chromosomes was observed among the progeny of mutant males. Two lines of evidence suggested that this chromosome loss was not due to errors in meiosis, but rather may be occurring during the early division in the embryo. First, only chromosome loss was observed and never nondisjunction, a phenotype not seen before for any known meiotic mutants. Second, preliminary examination of onion stage spermatids showed no evidence of meiotic chromosome loss (C. Davis and J. Tomkiel Dean, unpublished). The relatively low frequencies of loss observed genetically, however, did not preclude the possibility that meiotic loss was occurring at a frequency difficult to detect cytologically.

Here, we further investigate the nature of the *mcl(3)Z2566* mutant. We show the gene mutated by *mcl(3)Z2566* corresponds to *CG5538*. The expression pattern of the *CG5538* product in spermatogenesis suggests that it may be involved in the histone-to-protamine transition, and that *CG5538* function may be important for chromosome behavior subsequent to fertilization.

## CHAPTER II

### MATERIALS AND METHODS

#### Competent Cells

Competent cells were made according to the Scott-Simanis transformation protocol (M. Montiero, personal communication). Frozen DH5 $\alpha$  *E. coli* cells were streaked onto a  $\Psi\alpha$  plate (BactoYeast extract, BactoTryptone, MgSO<sub>4</sub>·7H<sub>2</sub>O, KCl, pH 7.6, BactoAgar) and grown overnight at 37°C. The next day, a single colony was used to inoculate 10 ml  $\Psi\beta$  medium (BactoYeast extract, BactoTryptone, MgSO<sub>4</sub>·7H<sub>2</sub>O, KCl, pH 7.6). The culture was grown overnight at 37°C at 250 rpm. It was transferred subsequently into 200ml  $\Psi\beta$  medium for further growth at 37°C at 250 rpm for a few hours until it reached an OD<sub>590</sub> of ~0.48. Cells were cooled on ice for 5 minutes then centrifuged at 6000 rpm for 5 minutes at 4°C. Cells were gently re-suspended in 100 ml of ice cold TfbI (30 mM potassium acetate, 100 mM RbCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 50 mM MnCl<sub>2</sub>·4H<sub>2</sub>O, 15% glycerol (v/v), pH 5.8) with a previously cooled pipette. After a 5 minute cooling on ice the cells were centrifuged at 6000 rpm for 5 minutes at 4°C. The pellet was then re-suspended in 12.5ml of ice cold TfbI (10 mM MOPS, 75 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 10 mM RbCl<sub>2</sub>, at pH 6.5, 15% glycerol (v/v)) and incubated on ice for 15 more minutes. 100  $\mu$ l aliquots were made and snap frozen in liquid nitrogen and stored at -80°C.

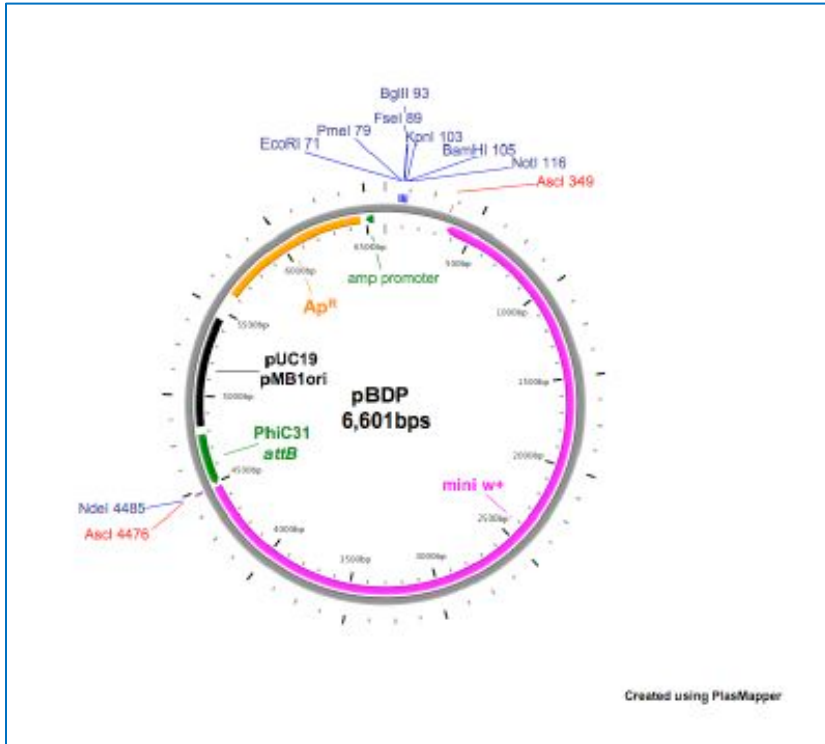
## **Bacterial Transformation**

Prior to transformation the competent DH5 $\alpha$  cells were thawed and kept on ice. 100 $\mu$ l cell aliquots were mixed with 50 ng of pBDP DNA (an ampicillin resistant vector) and were kept on ice for 20 minutes. The cells were heat-shocked at 43°C for 2 minutes and cooled on ice for 2 minutes before adding 1 ml LB (BactoYeast extract, BactoTryptone, NaCl, pH 7.0). The cells were incubated for 30 minutes at 37°C then plated on LB amp plates (BactoYeast extract, BactoTryptone, NaCl, BactoAgar, 100  $\mu$ g/ml Ampicillin pH 7.0) and incubated overnight at 37°C.

## **Vector Preparation**

Insect expression vector pBDP was selected for *in vivo* genomic targeting in *Drosophila melanogaster* embryos. The vector is 6601bp and contains a mini  $w^+$  gene for visibly identifying transformants, an extensive multiple cloning site (MCS) with restriction sites for *Eco*R1, *Kpn*1 and *Not*1, a *Phi*C31 attB site for targeted insertion and an ampicillin resistance gene for clone selection (Figure 2), (Rubin, Prideaux et al. 1985). A single colony from the transformation plate was used to inoculate 500ml LB-Ampicillin medium and was incubated overnight at 37°C at 250 rpm. The culture was centrifuged at 5000 rpm at 4°C for 5 minutes and the plasmid was purified from the cells using the Qiagen maxi plasmid kit (Qiagen, Valencia, CA). 5  $\mu$ l of the sample was run out on a 1% agarose gel with 10 $\mu$ l of 10 mg/ml ethidium bromide (EtBr) to verify the plasmid's presence. The DNA was quantified by absorbance at 260 nm using a Nanodrop device.

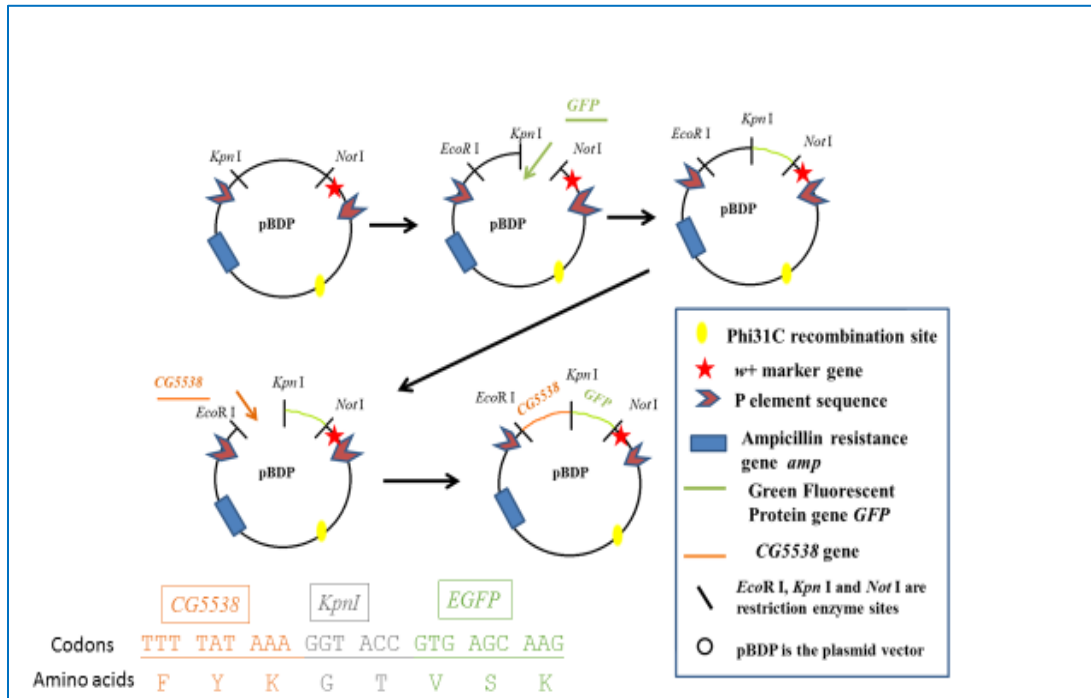
**Figure 2. General Structure of pBDP Cloning Vector.**



### **Cloning of *CG5538-EGFP* Fusion Gene into pBDP Vector**

The goal was to create a fusion gene encoding an enhanced version of the *A. victorea* Green Fluorescent Protein (EGFPS35T), (Tsien 1998) at the carboxy terminus of *CG5538*, (see Figure 3). To accomplish this, PCR primers were synthesized that allowed amplification of the *CG5538* promoter and coding region, lacking the stop codon (5538RKpnI) and amplification of the *EGFP* codons lacking the start codon (GFPFKpnI). Ligation of these PCR products into the vector created a fusion gene which encodes a *CG5538-GFP* fusion protein. The lack of a start *EGFP* codon ensured that any resulting green fluorescence could be attributed to the fusion protein rather than internally initiated translation of EGFP.

**Figure 3. Cloning of *CG5538* and *EGFP* Gene into pBDP Plasmid Vector.** The vector has an ampicillin resistance gene *amp* and P element ends flanking the multiple cloning site. The *EGFP* gene was cut with *Kpn* I and *Nor* I and ligated into pBDP that was cut with the same enzymes. *CG5538* was then ligated into pBDP+EGFP using *Eco*R I and *Kpn* I





### *PCR Amplification of EGFP DNA*

The *EGFP* gene was PCR amplified out of *pCasper HSGFP d-topors 1-1038FL* (Byungura 2009) using with a primer set with restriction enzyme restriction enzyme sites for *KpnI* (GFPR*KpnI*- AAGGTACCGTGAGCAAGGGCGAGGAG) and *NotI* (GFPR*NotI* - AAGCGGCCGCTCACTTGTACAGCTCGTC). PCR conditions were set at 30 cycles at 95°C for 30 seconds for denaturation, annealing at 54°C for 30 seconds and elongation at 72°C for 30 seconds. The primers were purchased from MWG Operon (Huntsville, AL). PCR products were purified and a 5 µl sample was purified on a 1% agarose gel with 10 µl of 10 mg/ml EtBr to confirm presence and size of DNA. For this and all subsequent gel purifications, the excised band was purified using GeneClean III Kit (MP Biomedicals LLC, OH).

### *Ligation of EGPF into Vector*

Purified pBDP vector was digested with *NotI* and gel purified in 1% agarose gel with 10 µl of 10 mg/ml EtBr. The band corresponding to 2.5kb was excised and gel purified. A second digestion with *KpnI* was set up and digested vector was gel purified. A 5µl sample was run out on a 1% agarose gel with 10 µl of 10 mg/ml EtBr to confirm presence and size of DNA. Vector DNA was quantified by absorbance at 260nm using a Nanodrop device.

The amplified *EGFP* DNA was digested separately with restriction enzymes *NotI* and *KpnI* (Promega, Madison, WI) at 37°C. DNA obtained from the first digestion with *NotI* was purified and the presence of the digested insert was verified by 1% agarose gel electrophoresis. A second digestion with *KpnI* was set up and the resulting DNA was

purified and the presence of *EGFP* insert was confirmed by 1% agarose gel electrophoresis. The *EGFP* fragment (20 ng), now cut with *NotI* and *KpnI*, and the pBDP vector (50 ng) previously digested with the same restriction enzymes were ligated together overnight at 15°C in 4 µl 10X T4 DNA Ligase Buffer with 1 µl of 3u/µl T4 DNA Ligase (Promega, Madison, WI). DH5α competent cells were transformed with the ligation product and were incubated on LB-amp plates overnight at 37°C. Resulting colonies were used to inoculate 5ml LB amp media overnight at 37°C in shaker at 250 rpm. To verify the presence of GFP in colonies with the vector, samples of each colony were PCR amplified individually with primers for GFP (GFPFK*pnl* – 5' AAGGTACCG TGAGCAAGGGCGAGGAG 3' and GFPR*NotI* – 5' AAGCGGCCGCTCACTTGT ACAGCTCGTC 3') and 10µl of the resulting DNA fragments were purified on a 1% agarose gel with 10 µl of 10 mg/ml EtBr. The plasmid DNA of each clone containing GFP was isolated using a CTAB Purification Protocol (Del Sal et al., 1988). To verify size of fragment 50 ng of DNA of each successful clone was digested with restriction enzymes *NotI* and *BglII* and purified on a 1% agarose gel containing 10 µl of 10 mg/ml EtBr. 500ml of LB-amp culture was grown overnight at 37°C in the shaker at 250rpm. The plasmid was purified using a Maxi-Prep DNA Purification Kit (Quiagen, Valencia, CA). The plasmid was quantified by absorbance at 260nm using a Nanodrop device. The clone was verified by sequencing of the plasmid DNA (MWG Operon Eurofins, Huntsville, AL)

### *Cloning of CG5538 Gene into Vector containing EGFP*

The vector containing GFP was digested with *Eco*RI and gel purified in 1% agarose gel with 10 µl of 10 mg/ml EtBr. DNA was purified with Geneclean III Kit (MP Biomedicals LLC, Ohio). The vector was then digested with *Kpn*I was and re-purified. A 5µl sample was separated by gel electrophoresis on a 1% agarose gel with 10 µl of 10 mg/ml EtBr to confirm the presence and size of DNA. Vector DNA was quantified by absorbance at 260nm using a Nanodrop device.

### *PCR Amplification of CG5538*

As a source of the *CG5538* gene, we obtained a bacteria artificial chromosome (BACPAC CH322-159P8) (CHORI, Oakland, CA). The *CG5538* gene was PCR amplified from the BACPAC with the primer set 5538FEcoRI (5' AAGAATTCGAGATTGAGGATGCTGTC 3') and 5538RKpnI (5' AAGGTACCTTTATAAAAATCATAATTAAGTGCGCTCTG 3') with the annealing temperature set at 54°C. The resulting PCR product was purified and digested first with *Eco*RI at 37°C, was purified again and then digested with *Kpn*I at 37°C. The fragments were gel purified on a 1% agarose gel with 10 µl of 10 mg/ml EtBr, excised from the agarose and purified using Geneclean III Kit.

### *Ligation of CG5538 into Vector with EGFP*

The *CG5538* fragment (20 ng) was ligated into 50 ng pBDP containing *EGFP* previously cut with *Eco*RI and *Kpn*I overnight at 15°C using 10X T4 DNA Ligase Buffer (4µl) and 3u/µl T4 DNA Ligase (1µl). DH5α cells were transformed with pBDP containing *CG5538-EGFP*. Transformed cells were plated out on LB amp plates. Plasmid DNA from clones was purified using CTAB/STET (Del Sal, Manfioletti et al. 1989). To

verify the plasmid a 50 ng sample was digested with *EcoRI* and *KpnI* to excise CG5538 and the digest was separated on a 1% agarose gel. DNA was quantified by absorbance at 260nm using a Nanodrop device and was sent in for sequencing to MWG Operon Eurofins, (Huntsville, AL). Once the construct sequences were verified by DNA sequencing, the vector DNA was sent in (Genetic Services Inc., Salisbury, MA) to be injected into 200 embryos to produce the first generation of transgenic flies (G<sub>0</sub>).

### **Fly Cultures**

Flies were reared at room temperature on standard cornmeal, molasses, agar media with propionic acid and tegasept added to prevent mold growth. All stocks were obtained from the Bloomington *Drosophila* Stock Center at Indiana University unless otherwise noted. Lines expressing siRNA were obtained from Vienna *Drosophila* Research Center in Vienna Austria. The *Df(3)desat*<sup>11573-C'1</sup> deletion was kindly provided by Dr. Jean- Francois Ferveur from the Universite de Bourgogne in Dijon, France.

### **Test for Effects on Sex and Fourth Chromosome Transmission**

Crosses of *y/y+Y* ; *spa* males either heterozygous or homozygous for *mcl(3)Z2566* alleles and *y w sn*; *C(4)cley* females were made and progeny phenotypes were scored up to 19 days after mating. Sex chromosome loss among progeny was detected as *y w sn* males, *yw//y+w* male mosaics, or *ywsn* male // *yw* female gynandromorphs. Diplo-exceptional sex chromosome progeny (i.e. those that received both the paternal X and Y) resulting from MI nondisjunction could be detected as *y+* females. Fourth chromosome loss was detected as *ci ey* progeny. Because of the variability in expressivity and penetrance of both the *ci* and *ey* markers, fourth

chromosome mosaicism could not be reliably assessed. Diplo-exceptional sperm could be detected as *spa* progeny.

### **Test for Effects on Major Autosome Transmission**

To test for autosomal chromosome loss or nondisjunction we set up the following crosses: five *C(2) EN bw sp* virgin females and 10 homozygous *mcl(3)Z2566* males and with five *C(3) st cu e* virgin females and 10 homozygous *mcl(3)Z2566* males. A total of 7 crosses were set up for each type with a total of 35 males and 70 females. Controls were set up in the same exact manner with *mcl(3)Z2566/Ser* flies. Progeny of these crosses survive only if they are euploid for the major autosomes, and such progeny only arise from paternal nondisjunction or loss. Surviving progeny of all crosses were counted. We used the metric of “progeny per male” as an indicator of nondisjunction or loss.

### **Establishment of Transgenic Fly Stocks**

Genetic Services injected 200 *white (w)* embryos with the transgene via site directed injections to an *attp40* landing site (kindly provided by the Perrimon Lab) at location 25C7. Of 200 injected *white (w)* embryos, 44 survived to adulthood ( $G_0$ ). Each  $G_0$  fly was crossed to  $w^{1118}$  flies to identify germline transformants based on transmission of the  $w^+$  transgene marker. Each male was crossed to 5  $w^{1118}$  female virgins and each female virgin was crossed to 3  $w^{1118}$  males. To establish independent transgenic stocks, a single generation 1 ( $G_1$ ) offspring exhibiting  $w^+$  activity was collected from each  $G_0$  cross and backcrossed to  $w^{1118}$  flies. The resulting  $w^+$  offspring of each separate line were intercrossed to create independent, homozygous transgenic stocks. The intensity of the

eye color was used as a guide to select for homozygosity for the transgene, (e.g. dark orange color was regarded as homozygous and light orange color as heterozygous).

## **Examination of Transgenic Flies**

### *Germline Expression of CG5538-EGFP*

Testes of transgenic flies were dissected in Schneider's medium (GIBCO BRL, Gaithersburg, MD), briefly fixed in 95% Ethanol (30 sec), stained with 1 µg/ml 4, 6-diamidino-2-phenylindole (DAPI) and mounted in phosphate buffered saline (PBS) on microscope slides. The testes were examined with Olympus Fluoview FV500 confocal laser scanning microscope for GFP fluorescence.

Testes from *H2Av-RFP/ CG5538-GFP* males were examined to define *CG5538* expression and localization relative to the histone-to-protamine transition.

### *Transgene Rescue*

To test for the ability of the transgene to complement the *mcl(3)Z2566* mutant, selected inserted transgenes (lines 3 and 22) were crossed into a *yw/y+Y; mcl(3)Z2566/ Df(3)desat<sup>11573-C'1</sup>; spa<sup>pol</sup>* background. Males bearing zero (control), one or two copies of the transgene were crossed to *y w sn; C (4) ci ey* females to test for chromosome loss as above.

### *Inhibition of CG5538 by RNAi*

*CG5538* (*CG5538*<sup>P{KK106939}</sup>) and *desat1* (*Desat1*<sup>P{KK107747}</sup>) RNAi constructs were purchased from Vienna *Drosophila* Research Center in Vienna Austria. These express hairpin RNAs (which are processed into siRNAs) under the control of the GAL4-inducible *UAS* promoter. A Gal4-expressing driver line, *T76* (Hrdlicka, Gibson et al.

2002; Arya, Lodico et al. 2006) was used to drive expression of the siRNAs in the male germline.

To verify RNAi knockdown, *T76;CG5538-GFP/ CG5538<sup>P{KK106939}</sup>* testes were dissected in Schneider's medium, fixed 30 sec in 95% ethanol and observed for GFP fluorescence using confocal microscopy. *T76; CG5538-GFP* males (without the RNAi construct) served as controls. Subsequently *T76 Gal4* driver males were mated to *CG5538<sup>P{KK106939}</sup>* and *Desat1<sup>P{KK107747}</sup>* virgin female. Male progeny resulting from these crosses were crossed to *y w sn : ci ey* virgin females. The offspring was scored and the frequencies of fourth chromosome loss were measured as above.

### **Generation of New *CG5538* Alleles by P-Element Transposition**

A P element inserted into the 3' untranslated region of *CG5538(P{EP}CG5538<sup>G1987</sup>)* was mobilized using the transposase source  $\Delta 2-3$  (Laski, Rio et al. 1986) to generate new insertions and/or deletions. Despite its position, this insertion was found to complement both *mcl(3)Z2566* mutation and the *Df(3)desat11573-C'1* deletion (Marcillac, Bousquet et al. 2005). Individual *yw/Y; cn bw; Sb e  $\Delta 2-3$ /P{EP}CG5538<sup>G19873</sup>* males were crossed to 5 *y w; st mcl(3)Z2566 Sb/TM3, ser; spa* female virgins. Progeny with either white eyes (*w*-) and/or orange (*w*+) eyes that were distinguishably different in coloration from the original *P{EP}CG5538<sup>G19873</sup>* were selected. To ensure independence, only one progeny was selected from each parent male. A total of 200 progeny were selected: 165 white [*w*<sup>-</sup>] and 20 orange eyed [*w*<sup>+</sup>]. Potential new mutations were tested for complementation of *mcl(3)Z2566*, and stocks established

from the two that failed to complement: (*P{EP}CG5538<sup>G19873m1</sup>* (*w<sup>-</sup>*) and *P{EP}CG5538<sup>G19873m2</sup>* (*w<sup>+</sup>*).

#### *DNA Sequencing of New CG5538 Alleles*

Genomic DNA from *P{EP}CG5538<sup>G19873m2</sup>* male flies was extracted according to the protocol by E. Jay Rehm (Berkeley Drosophila Genome Project). Briefly, for each genotype about 30 flies were ground up in Buffer A (Tris pH 7.5, EDTA, NaCl, SDS) and incubated on ice in an 1: 2.5 LiCl:KAc solution. Samples were centrifuged at 13,000 rpm for 10 min and the lysate was extracted with Phenol/Chloroform. Genomic DNA was precipitated by the addition of 0.7 volumes Isopropanol and centrifuged 15 min at 13,000 rpm. After resuspension in distilled water, the DNA was quantified by absorbance at 260nm using a Nanodrop device. DNA was sequenced by MWG Operon Eurofins, (Huntsville, AL).

#### *PCR Amplification*

*CG5538* was PCR amplified from *P{EP}CG5538<sup>G19873m2</sup>* flies using following primer sets: CG5538F1/CG5538R1; CG5538F2/CG5538R2; CG5538F3/CG5538R3; CG5538F4/CG5538R4 (Table 1) with annealing temperature at 52°C. Amplified fragments were gel purified on 2% agarose gels with 10µl of 10 mg/ml EtBr, excised from the gel and purified with a GeneClean III Kit (MP Biomedicals LLC, Ohio). Fragments were sequenced by MWG Operon Eurofins Genomics ( Huntsville, AL) using the following primers: CG5538F1/R1; CG5538F2/R2; CG5538F3/R3 CG5538F4/R4 for *P{EP}CG5538<sup>G19873m2</sup>* and CG5538F5/R1; CG5538F5/R4.2; CG5538F5/R4; CG5538F4/CG5538RNotI; CG5538F4.1/R4.3 for *P{EP}CG5538<sup>G19873m1</sup>* (Table 1).



### *Recombination Mapping*

For the  $P\{EP\}CG5538^{G19873m2}$  chromosome, both the  $w+$  phenotype and the failure to complement  $mcl(3)Z2566$  were mapped by recombination with respect to the third chromosome dominant markers *Kinked* (*Ki*), *Ultrabithorax* (*Ubx*) and *Drop* (*Dr*). 32 recombinant chromosomes from  $P\{EP\}CG5538^{G19873m2}/Ki\ Ubx\ Dr$  female were recovered *in trans* to the  $Df(3)desat1^{1573-C'1}$  deletion. Offspring of test males were scored for chromosome loss and for the presence of the mini  $w+$  gene by crossing to  $y\ w\ sn; C$  (4) *ci ey* females and scoring for  $w+$  and/or *ci ey* progeny.

**Table 1. Primers for Sequencing of New CG5538 Alleles**

F1	AATCATGGGAGGGTTTAG	R1	GTGTTCTAACATCGATCG
F2	TAGTATTTAGCGTAAGCC	R2	CCATACACTATGGTGCTA
F3	GTCTGCGCTAATGCAATC	R3	TTGGTATAGGTCCATCCC
F4	AATGGCCACCACCAGTAT	R4	GGACTCTCGTTGTATTAG
F4.1	ATCGTCAATAGGTAGCTG	R4.2	GCAGCGCGATAATAACAG
F5	AAGCAAGCAAGCCAGCC AT	R4.3	GCGTTGTTGTTGACATCC
		RNotI	CAGAGCGCACTTAATTATGATTTT TATAAA

## CHAPTER III

### RESULTS

#### Genetic Characterization of the *mcl(3)Z2566* Chromosome Transmission Defect

##### *Sex and Fourth Chromosome Loss*

To ask if the *mcl(3)Z2566* affected sex chromosomes as well as fourth chromosomes, and to quantify both loss and nondisjunction, we examined males homozygous for the original mutation (Wakimoto, Lindsley et al. 2004). In addition, to rule out effects of other recessive mutations that might be present on the *mcl(3)Z2566* chromosome, and to ask if *mcl(3)Z2566* behaved as a null allele, we also examined trans-heterozygotes bearing the original mutation *in trans* with a deletion, *Df(3)desat1<sup>1573C'1</sup>* (Marcillac, Bousquet et al. 2005). In previous mapping experiments, this deletion was shown to fail to complement the *mcl(3)Z2566* fourth chromosome loss phenotype (C. Davis, J. Chmielewski and J. Tomkiel Dean, unpublished).

Males were crossed to *y w sn; C (4) ci ey* females. From each cross progeny were simultaneously assessed for 4<sup>th</sup> chromosome and sex chromosome loss and/or nondisjunction (see Materials and Methods). As controls we scored the progeny of *Df(3)desat1<sup>1573C'1</sup>/TM3, Ser* and *mcl(3)Z2566/TM3,Ser* flies. Approximately 8% of progeny of homozygous *mcl(3)Z2566* males and 12% of the progeny of *mcl(3)Z2566/Df(3)desat1<sup>1573C'1</sup>* males lacked a paternal fourth chromosome. We also observed 2.1% and 1.2% sex chromosome loss from these respective crosses. The frequencies of fourth

and sex chromosome loss among progeny of *mcl(3)Z2566/ Df(3)desat1<sup>1573C'1</sup>* males were comparable, suggesting that this relatively low level of loss is the null phenotype. Frequencies of simultaneous sex and fourth chromosome loss (0.2%) were close to that predicted by independence, suggesting that the behavior of the sex and fourth chromosomes did not influence each other. We did not see any increase in diplo-exceptional progeny over control crosses (i.e. that inherited 2 copies of a paternal chromosome), suggesting that the null-exceptional progeny were the result of chromosome loss rather than nondisjunction. In control crosses involving heterozygous males, the frequencies of chromosome loss were less than 0.5%, indicating that the mutation *mcl(3)Z2566* is recessive.

These tests do not distinguish between pre-meiotic, meiotic or post-fertilization paternal chromosome losses. However, we also observed rare sex chromosome mosaic offspring from these crosses, which were never seen in control crosses. This suggests that at least some chromosome loss occurs during the early mitotic divisions in the embryo. For both the homozygous *mcl(3)Z2566* and *mcl(3)Z2566/Df(3)desat1<sup>1573C'1</sup>* a total of 14 sex chromosome mosaics were found (10 XX//XO and 4 XY//XO) (see Table 2), indicating that both the X and Y are affected.

### *Major Autosome Loss*

To ask if *mcl(3)Z2566* similarly caused loss of the major autosomes, we crossed mutant males to females bearing compound autosomes (*C(A)*). From such crosses, progeny survive only if they receive either two or no copies of the corresponding

autosome from the father. As a metric of chromosome loss, we counted the number of viable progeny produced per male. In crosses to 70 *C(2)* females, no progeny were produced from 35 homozygous *mcl(3)Z2566* males, versus three from *mcl(3)Z2566 Sb/Ser* males. Similarly in crosses to *C(3)* females, only one offspring was produced from 35 homozygous males versus none from *mcl(3)Z2566/Ser* control males. These results suggest that the mutation does not significantly increase autosomal loss or nondisjunction, however we cannot rule out that there may be very low levels of autosome loss induced.

**Table 2. Sex and Fourth Chromosome Disjunctional Data.** Collected from crosses of  $y/y^+Y$ ;  $CG5538$ ;  $spa^{pol}$  males to  $y w sn$ ;  $C(4)RM ci ey /0$  females.

Recovered male gametes:	$Y;4$	$X;4$	$0;4$	$X/Y;4$	$X;0$	$Y;0$	$X;4/4$	$Y;4/4$	$0;0$	$0;4/4$	$X/Y;0$	$X/Y;4/4$
Paternal Genotyp												
$mcl(3)Z2566$	1114	1798	59	0	144	109	0	3	8	0	0	0
$000mcl(3)Z2566/Df(C'1)$	1101	1804	23	0	188	157	1	2	13	0	0	0
$mcl(3)Z2566/TM3, Ser$	1903	2616	3	1	3	1	0	1	2	0	0	0
$Df(C'1)/TM3, Ser$	409	728	2	0	1	0	1	0	0	0	0	0
$CG5538^{P8}$	164	346	9	0	57	25	0	0	2	0	0	0
$CG5538^{P8}/TM3, Ser$	751	1013	0	0	2	2	1	0	0	0	0	0
$CG5538^{P39}/Df(C'1)$	483	1053	7	0	48	30	1	0	0	0	0	0
$CG5538^{P39}/TM3, Ser$	318	410	0	0	1	0	0	0	0	0	0	0

	nullo XY	diplo XY	nullo 4	diplo 4	nullo XY + nullo 4*	Mosaics XX//XO XY//XO	
<i>mcl(3)Z2566</i>	2.1	0	8.0	0.1	0.2 (0.2)	5	3
<i>mcl(3)Z2566/ Df(C'1)</i>	1.2	0	12.4	0.1	0.5 (0.2)	5	1
<i>CG5538<sup>P8</sup></i>	1.8	0	13.9	0	0.3 (0.3)	2	2**
<i>CG5538<sup>P39</sup>/Df(C'1)</i>	0.4	0	4.8	0.1	0 (0.04)	2	0
<i>mcl(3)Z2566/TM3, Ser</i>	0.1	0	0.1	0	0		
<i>CG5538<sup>P8</sup>/TM3, Ser</i>	0	0	0.1	0.1	0	1	0
<i>CG5538<sup>P39</sup>/TM3, Ser</i>	0	0	0.1	0	0		
<i>Df(C'1)/TM3,Ser</i>	0.2	0	0.1	0.1	0		

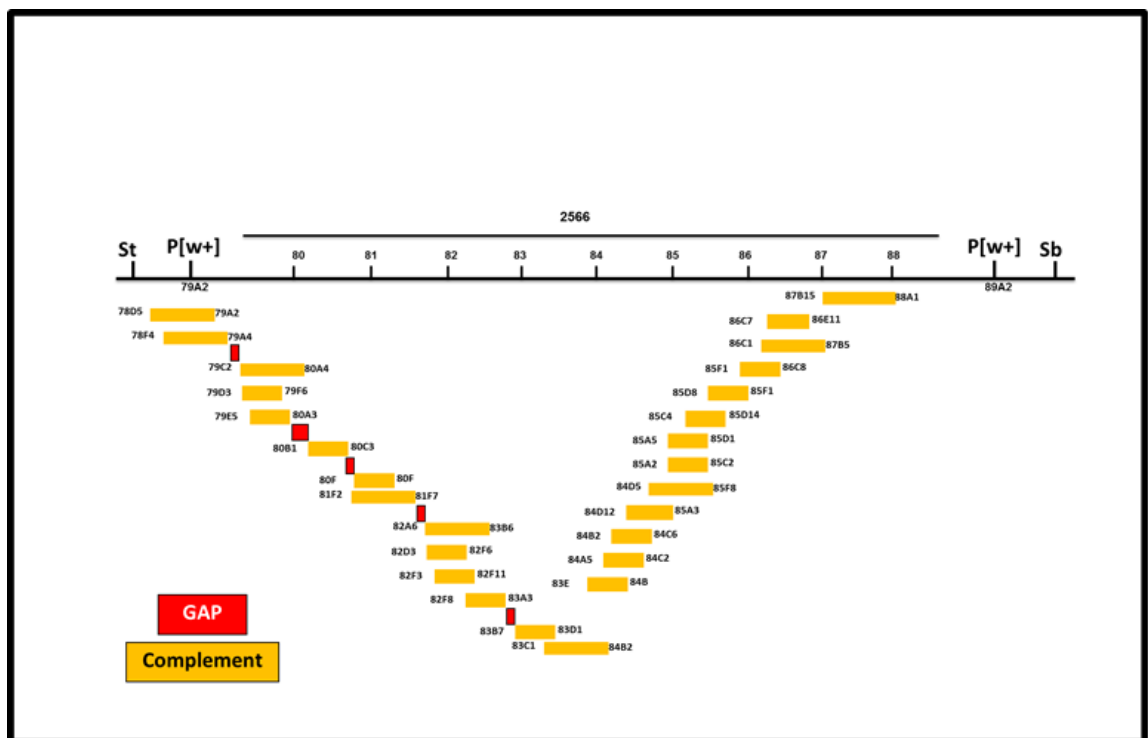
\*Frequencies of simultaneous sex and fourth chromosome nondisjunction. Observed and (Expected based on independence).

\*\* One was mosaic for sex chromosome and also had chromosome 4 loss. New alleles: *CG5538<sup>P39</sup>* (*P{EP}CG5538<sup>G19873m1</sup>*) and *CG5538<sup>P8</sup>* (*P{EP}CG5538<sup>G19873m2</sup>*). *Df(3)desat1<sup>1573-C'1</sup>* is abbreviated as *Df(C'1)*

### Mapping of *mcl(3)Z2566*

The *mcl(3)Z2566* mutation had been mapped by recombination to salivary gland bands 86-87 on the short arm of chromosome 3 (Figure 4) (C. Davis, J. Chmielewski and J. Tomkiel Dean, unpublished).

**Figure 4. Deletion Mapping of the Salivary Gland Chromosome Region 87-86.** Yellow indicates regions that showed complementation. Red indicates gap regions that were not tested (C. Davis, J. Chmielewski and J. Tomkiel Dean, unpublished).

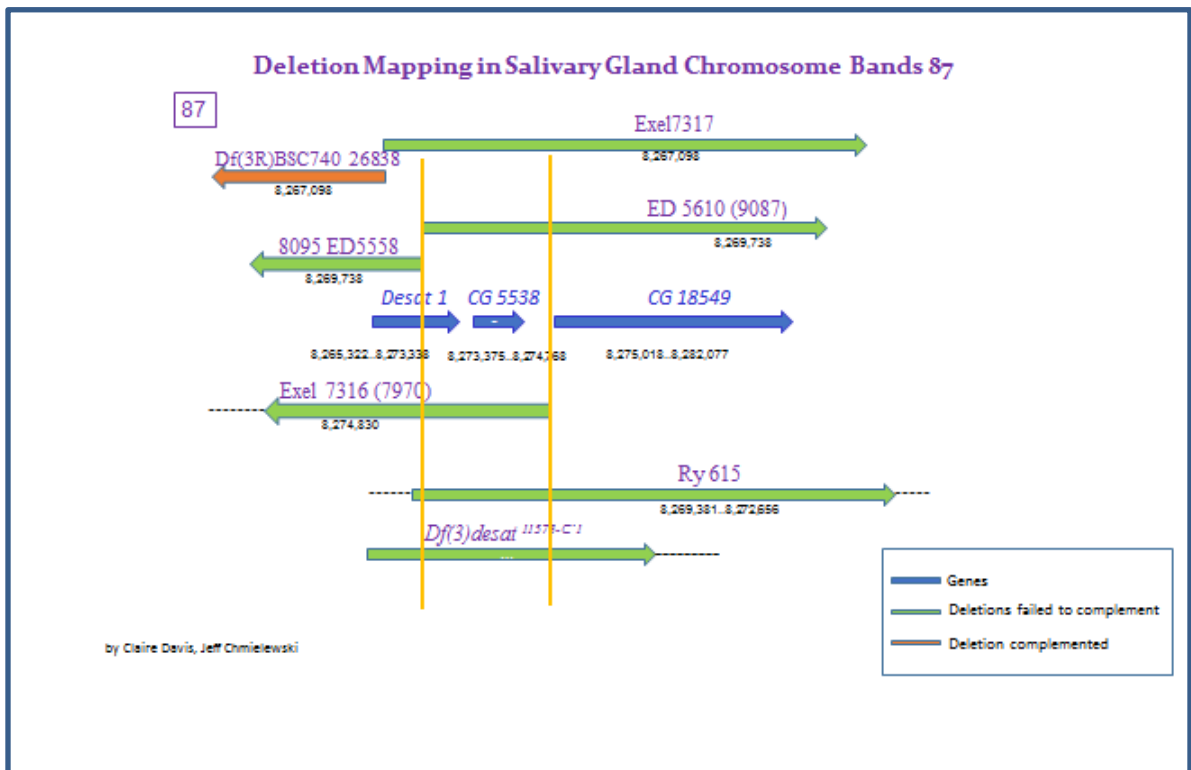


Further deletion mapping initially suggested that the candidate gene was *desat 1* (Figure 5). However sequencing of the *desat1* gene from homozygous *mcl(3)Z2566* DNA showed no differences from the wildtype progenitor chromosome. In contrast, the mutant DNA revealed a missense A(89)T mutation in the adjacent gene, *CG5538*. This results in the



replacement of the charged, hydrophilic Aspartic acid (D) to a non-polar, hydrophobic Valine (V), (D2V) (Figure 6) (C. Davis, J. Chmielewski and J. Tomkiel Dean, unpublished). This suggested that the complementation results with *Df(3)ED5558* may be in error, that the *Df(3)ED5558* chromosome may harbor a *CG5538* mutation, or that the deficiency results in a position-effect on *CG5538*.

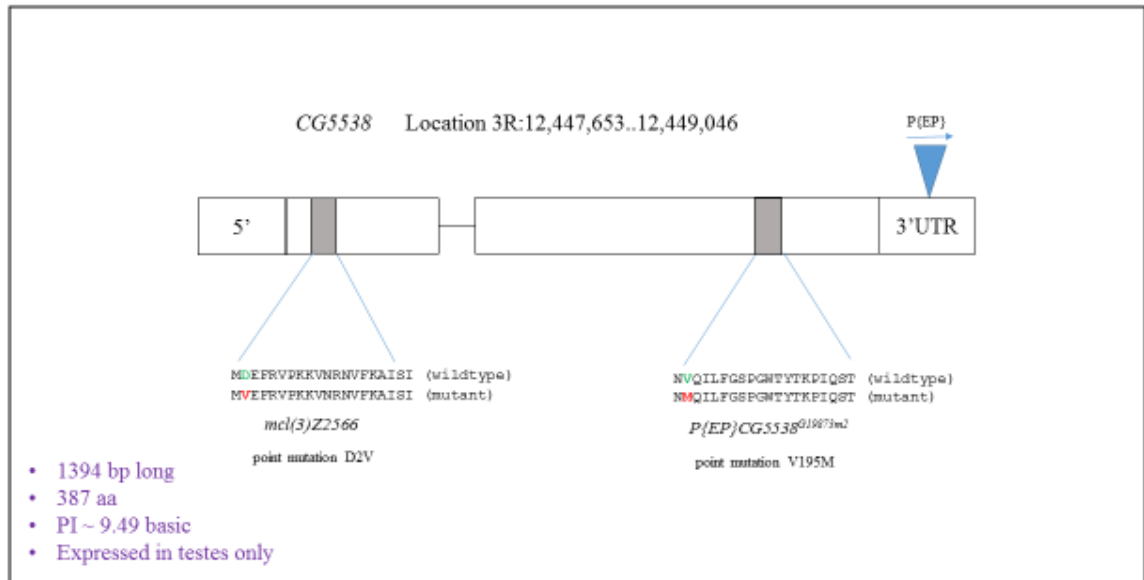
**Figure 5. Deletion Mapping in Salivary Gland Chromosome Band 87.** Blue arrows: location, size and direction of the three genes at chromosome band 87 (*Desat 1*, *CG5538* and *CG18549*). Green arrows: deletions and their directions at chromosome band 87.



## Identifying *CG5538* as the Mutated Gene

We used three different approaches to verify that the mutation in *CG5538* corresponds to *mcl(3)Z2566*. First, we created transgenic flies expressing an *EGFP*-labeled copy of wildtype *CG5538* and tested whether the transgene rescued.

**Figure 6. Diagram of *D. melanogaster* Gene *CG5538* and Mutations.** Insertion of P-elements is indicated by blue triangles. Gray boxes show excerpts of the protein sequences of the wildtype and the mutant alleles *mcl(3)Z2566* and *P{EP}CG5538<sup>G19873m2</sup>*. *mcl(3)Z2566*: missense mutation A(89)T changes aspartic acid (D) to Valine (V). *P{EP}CG5538<sup>G19873m2</sup>*: point mutation G(691)A changes Valine to Methionine.



Secondly, we inhibited *CG5538* expression with siRNA. And thirdly, we generated new *CG5538* alleles by P element transposition and tested them for chromosome loss.

### *Transgene Rescue of CG5538*

We created transgenic flies by targeted integration of *CG5538-EGFP* fusion gene into chromosome 2L at location 25C7 (Genetic Services). We established 10 independent transgenic lines, and chose one at random to test the ability of the transgene to complement the *mcl(3)Z2566* mutation. The transgene was crossed into a *yw/y+Y; mcl(3)Z2566 / Df(3)desat<sup>11573-C'1</sup>; spa<sup>pol</sup>* background. Males bearing zero (control), one or two copies of the transgene were crossed to *y w sn; C (4) ci ey* female virgins to test for chromosome loss (Table 3). Transgenic brothers heterozygous for each of the two alleles, i.e. *Df(3)desat<sup>11573-C'1</sup> / TM3, Ser* and *mcl(3)Z2566 / TM3, Ser*, served as controls. Progeny of males with no copies of the transgene exhibited 0.9 percent X chromosome loss and 8.2 percent 4<sup>th</sup> chromosome loss, consistent with previous results. Offspring of males with one or two copies of the transgene showed significantly reduced levels of both sex (0%) and fourth chromosome loss (0.6 and 0.5%, respectively). This shows that the transgene rescued the mutant phenotype and supports the hypothesis that the causative mutation *mcl(3)Z2566* lies in *CG5538*.

**Table 3. Rescue by CG5538-EGFP Transgene**

Paternal Genotype	# paternal copies of transgene		Recovered male gametes								
			Y;4	X;4	0;4	X;0	Y;0	X;4/4	Y;4/4	0;0	0;4/4
<i>mcl(3)Z2566/ Df(3)C'1</i>	0		831	1093	17	97	78	2	1	4	0
<i>mcl(3)Z2566/TM3, Ser</i>	0		440	437	0	0	0	0	0	0	0
<i>Df(3)C'1/TM3,Ser</i>	0		416	540	0	1	0	0	0	0	0
<i>mcl(3)Z2566/ Df(3)C'1</i>	1	w	438	579	0	1	5	0	1	0	0
		w+	482	629	0	2	4	0	0	-	-
<i>Df(3)C'1/TM3,Ser</i>	1	w	203	267	0	0	0	0	2	0	0
		w+	209	235	0	0	0	1	1	-	-
<i>mcl(3)Z2566/TM3, Ser</i>	1	w	270	307	0	1	0	1	0	0	0
		w+	211	278	0	2	0	0	0	-	-
<i>mcl(3)Z2566/ Df(3)C'1</i>	2		940	1285	0	1	0	0	0	-	-
<i>Df(3)C'1/TM3,Ser</i>	2		267	370	0	0	0	0	0	-	-
<i>mcl(3)Z2566/TM3, Ser</i>	2		57	513	0	0	0	0	0	-	-

% Chromosome loss	# paternal copies of transgene		ullo XY	ullo 4	ullo XY + ullo 4*	Mosaics
<i>mcl(3)Z2566/ Df(3)C'1</i>	0		0.9	8.2	0.19 (0.07)	0.3
<i>mcl(3)Z2566/ Df(3)C'1</i>	1	w	0	0.6	0	0
		w+	0	0.5	0	0
<i>mcl(3)Z2566/ Df(3)C'1</i>	2		0	0	0	0

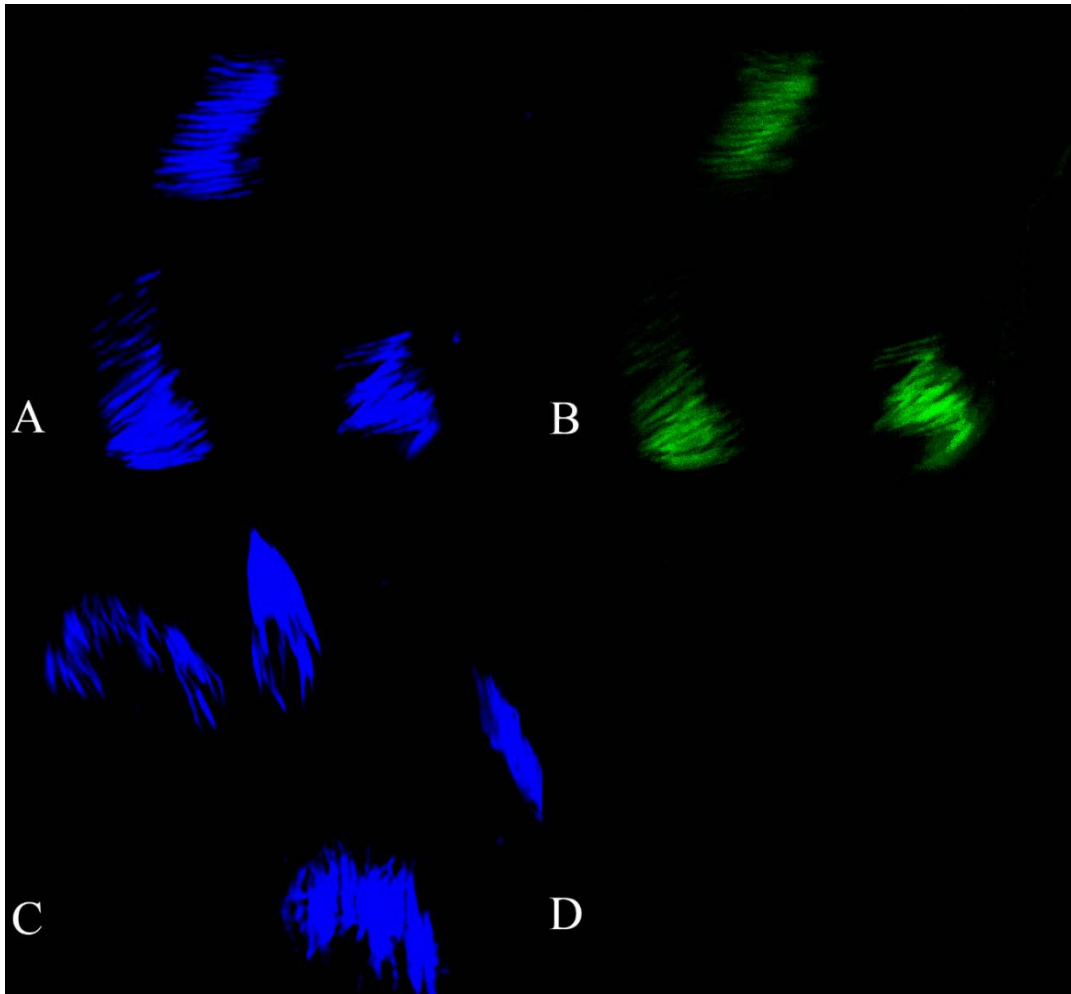
\*Frequencies of simultaneous sex and fourth chromosome nondisjunction. Observed and (Expected based on independence).  
*Df(3)desat1<sup>1573-C'1</sup>* is abbreviated as *Df(3)C'1*.

### *RNAi Knockdown of CG5538*

As another line of evidence that *mcl(3)Z2566* affects *CG5538*, we set up an experiment to knock down the expression of *CG5538*. Flies bearing a GAL4-inducible UAS-RNAi transgene specifically targeting *CG5538* mRNAs were mated to flies that carry a transgene that expresses the transcriptional activator Gal4 under the control of a tissue specific promoter (a “driver” line). We chose *T76* as the driver due to its expression in male germ line at or prior to the earliest stages of primary spermatocytes (Hrdlicka, Gibson et al. 2002), (Arya, Lodico et al. 2006).

To verify RNAi knockdown, *T76;CG5538-EGFP/CG5538<sup>P{KK106939}</sup>* dissected testes were observed for a reduction in GFP signal using confocal microscopy. *T76;CG5538-EGFP* males (without the RNAi construct) served as controls. We failed to detect any GFP signal in males expressing the RNAi construct, indicating that remaining fusion protein was reduced below detectable levels (Figure 7). We then tested both *T76/CG5538<sup>P{KK106939}</sup>* and *T76/Desat1<sup>P{KK107747}</sup>* males for chromosome loss. Males were crossed to *y w sn : ci ey* virgin females, and the frequencies of fourth chromosome loss were determined among the offspring. We observed approximately 2% 4<sup>th</sup> chromosome loss among progeny of *T76/CG5538<sup>P{KK106939}</sup>* males, while progeny of *T76/Desat1<sup>P{KK107747}</sup>* allele had only background levels of loss (~ 0.1%), (Table 4).

**Figure 7. RNAi Knockdown of *CG5538-EGFP* Transgene Expression Levels in Testes.** A. DAPI staining and B. GFP signal in sperm nuclei of male flies homozygous for the *CG5538-EGFP* transgene. C. DAPI staining and D. GFP signal in sperm nuclei of *T76;CG5538-GFP/CG5538<sup>P[KK106939]</sup>* flies.



**Table 4. 4<sup>th</sup> Chromosome Loss by RNAi Inhibition of *CG5538* and *desat1***

Gal4 driver	Expression	RNAi Construct	Target Gene	% nullo-4	Total
None		P{KK106939}	<i>CG5538</i>	0.08	1281
		P{KK107747}	<i>desat1</i>	0.00	1144
<i>T76</i>	CC, G, S1-2	P{KK106939}	<i>CG5538</i>	1.86**	1024
		P{KK107747}	<i>desat1</i>	0.12	807

G= gonial cells, S= spermatocyte, CC = cyst cell \*\*p<0.01

#### *New CG5538 Alleles*

A P element inserted into the 3' untranslated region of *CG5538*, (*P{EP}CG5538<sup>G1987</sup>*), was mobilized using the transposase source  $\Delta 2-3$  to generate new insertions and/or deletions (see Methods). Two new *CG5538* alleles (*P{EP}CG5538<sup>G19873m1</sup>*(w<sup>-</sup>) and *P{EP}CG5538<sup>G19873m2</sup>*(w<sup>+</sup>) were identified. (*P{EP}CG5538<sup>G19873m1</sup>*(w<sup>-</sup>) was not viable as a homozygote, but was tested *in trans* with *Df(3)desat<sup>11573-C'1</sup>* and showed both sex and fourth chromosome loss, albeit at lower frequencies than the original *mcl(3)Z2566* allele (Table 2). This may indicate that this allele is hypomorphic. The homozygous *P{EP}CG5538<sup>G19873m2</sup>* allele showed a 13.9% fourth chromosome loss and 1.8% sex chromosome loss. These results were similar to the results of the original mutation at 8% 4<sup>th</sup> and 2.1% sex chromosome loss, indicating that this allele is also likely a null allele (Table 2). Together these results suggest that the *CG5538* gene is disrupted in the new alleles.



### Molecular Characterization of $P\{EP\}CG5538^{G19873m2}$

Sequencing of allele  $P\{EP\}CG5538^{G19873m2}$  revealed that the P element excised precisely from  $CG5538$ , but a missense mutation in the  $CG5538$  coding region was present at G(691)A. Conceptual translation of the amino acid sequence revealed a substitution of V(195)M. This chromosome retained the P element-associated [*w+*] marker, but there was no evidence of P element sequences associated with the  $CG5538$  locus, indicating that the original P element has transposed to a new location on chromosome 3.

The other transposase-induced allele,  $P\{EP\}CG5538^{G19873m1}$ , has not yet been molecularly characterized.

### Recombination Mapping

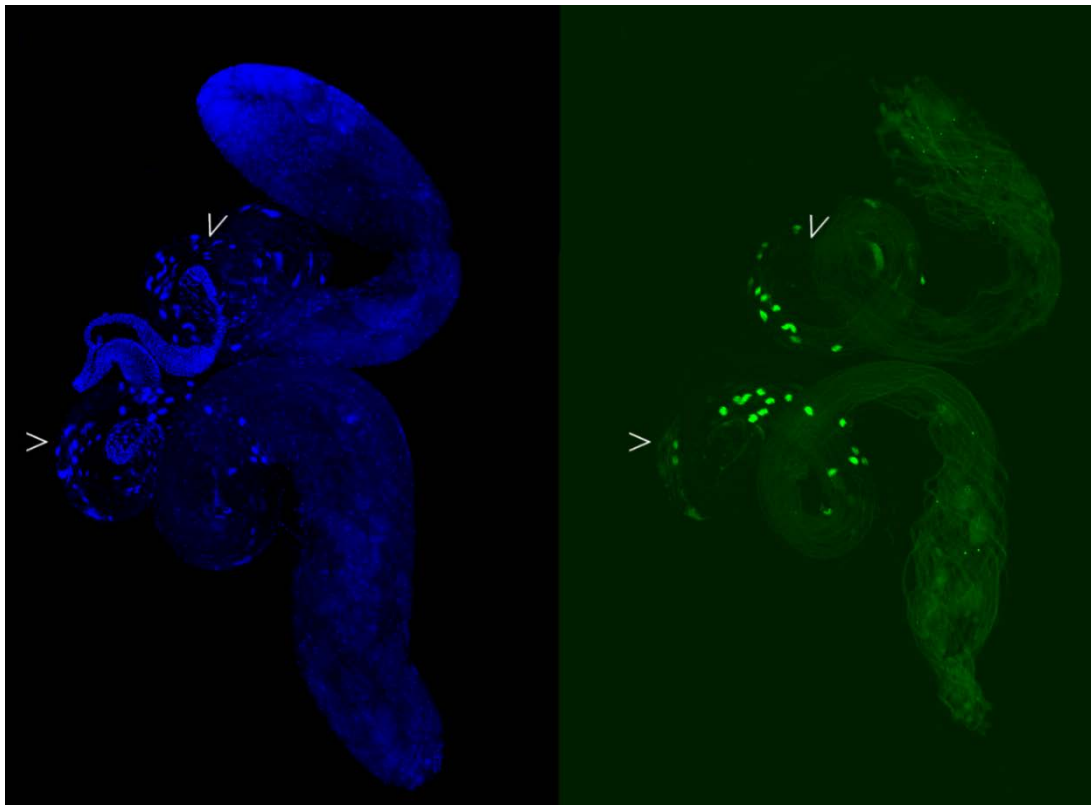
For the  $P\{EP\}CG5538^{G19873m2}$  chromosome, both the *w+* phenotype and the failure to complement *mcl(3)Z2566* were mapped by recombination between the third chromosome dominant markers *Kinked* (*Ki* at 83D-83E) and *Ultrabithorax* (*Ubx* 89D6-89D9), consistent with the location of  $CG5538$  at 87B11.

### **Expression of $CG5538$**

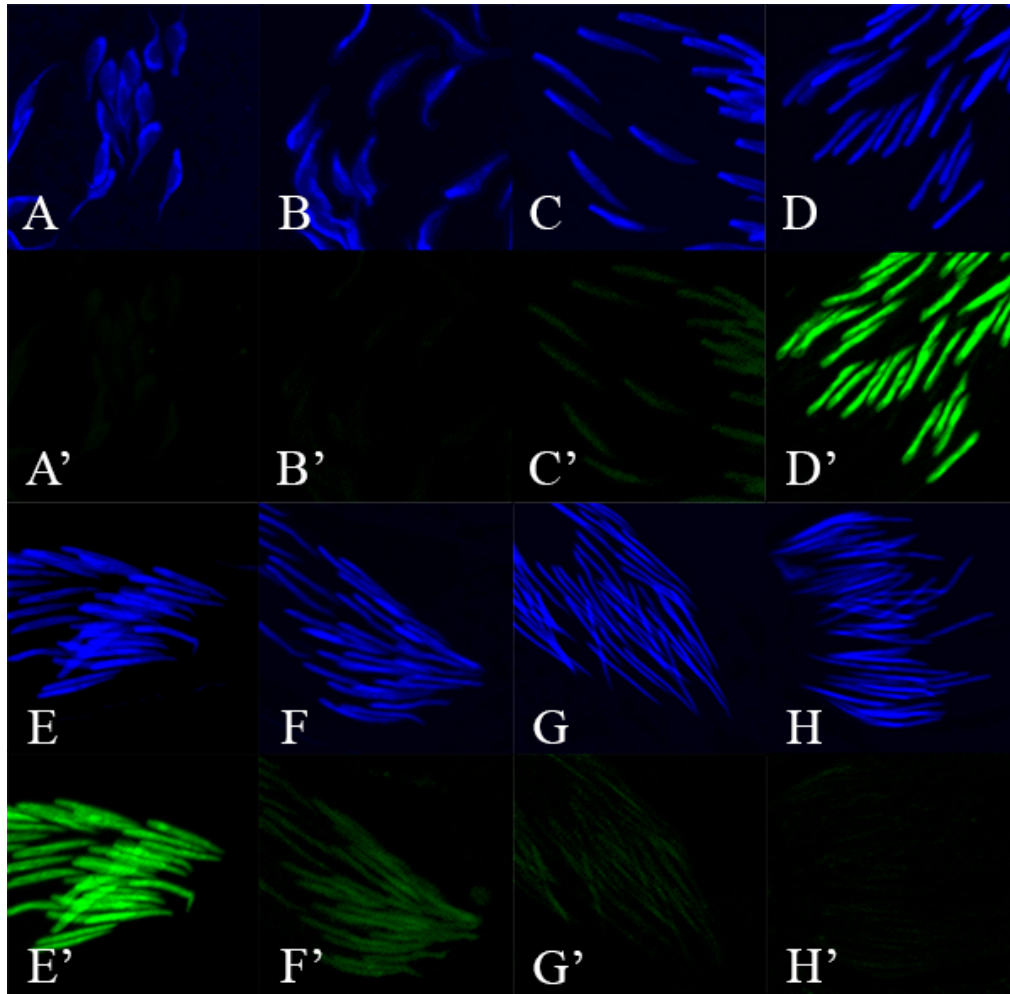
Testes of  $CG5538$ -EGFP transgenic flies were squashed and examined by confocal laser microscopy for GFP signals at different stages of spermatogenesis (Figures 8 and 9). No expression of  $CG5538$  was observed in spermatogonia or spermatocytes.

The earliest stage at which GFP could be detected was at the early canoe stage or spermatid differentiation, a stage at which the nuclei are condensing as histones are gradually getting replaced by protamines. The intensity of the green GFP signal was progressively increased at the mid canoe stage and gradually declined in late canoe stage. At the needle stage, when the nuclei were maximally condensed, the GFP signal was no longer detectable.

**Figure 8. Whole Mount Transgenic Testes.** Blue image shows DAPI staining of sperm nuclei. Green image shows *CG5538-EGFP* transgene expressed in late canoe stage. Arrows point to mature sperm that lack detectable signal.



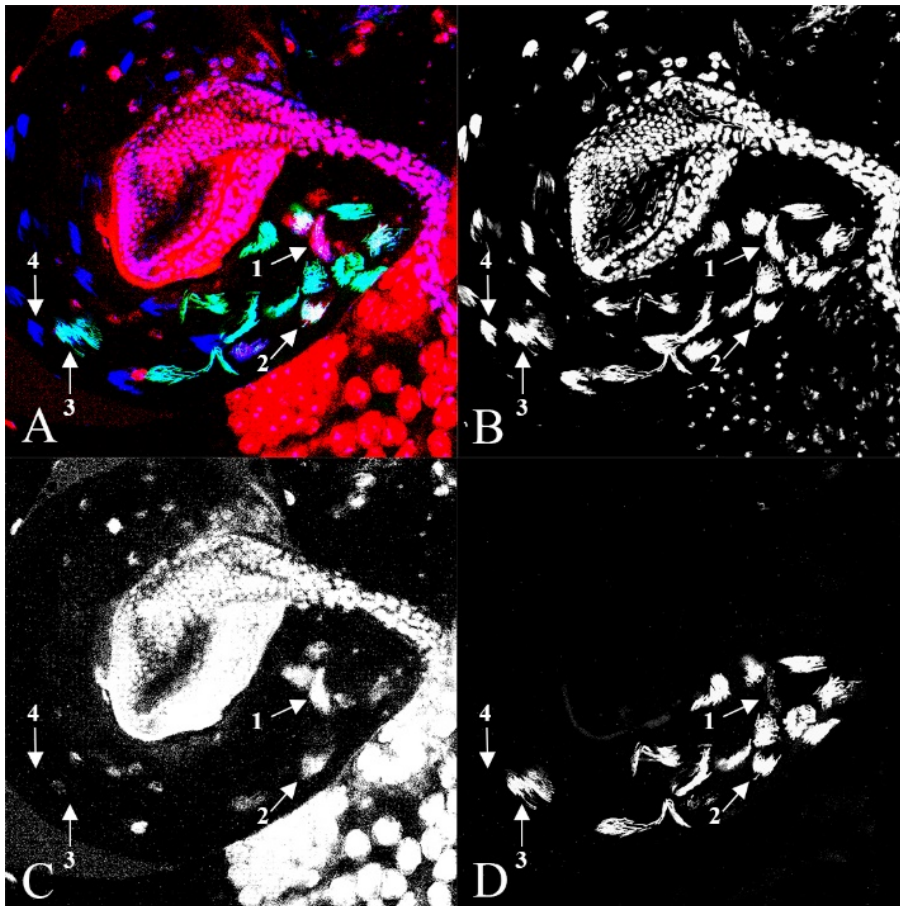
**Figure 9. Squashed Preparation of *Drosophila* Testes Showing Spermatid Developmental Stages and CG5538-EGFP Expression.** DAPI-stained DNA (blue), CG5538-EGFP (green). **A (A') and B (B')**. Leaf stage. CG5538 is not expressed. **C and C'**. Early canoe stage. Sperm begins to condense. Low level expression of CG5538. **D (D') and E (E')**. Mid canoe stage. Strong expression of CG5538. **F (F') and G (G')**. Late canoe stage and transitioning into individualized mature sperm. Expression of CG5538 declines. **H and H'**. Needle shape. Fully matured individualized sperm nuclei. CG5538 is not detectable.



During spermatogenesis chromatin is reorganized post meiosis by replacing histones with protamines to allow for compacting of sperm nuclei. (Gartner, Rothenbusch et al. 2015).

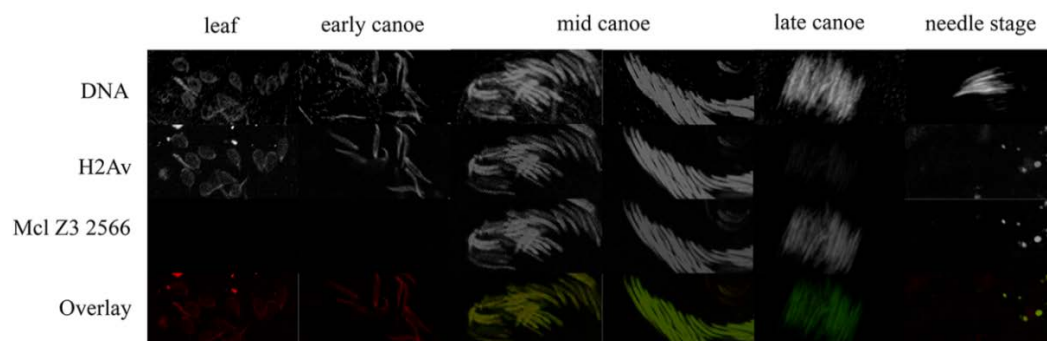
To define *CG5538* expression relative to the histone-protamine transition, we examined testes from *H2aV-RFP/CG5538-GFP* males (Figure 10).

**Figure 10. Squashed Preparation of *Drosophila* Testes Expressing both H2aV-RFP and CG5538-EGFP Fusion Proteins. (A) Overlay of DAPI stained DNA (blue), H2Av-RFP (red) and CG5538-EGFP (green). (B) DAPI stained DNA nuclei at all stages of spermiogenesis. (C) Expression of H2Av-RFP. (D) Expression of CG5538-EGFP. 1. Early canoe stage. Only H2Av-RFP present. 2. Mid canoe stage. Both H2Av-RFP and CG5538-EGFP present. 3. Late canoe stage. Only CG5538-EGFP present. 4. Mature sperm CG5538-EGFP absent.**



H2Av (Histone 2A variant) is the *Drosophila* version of histone H2A found in human chromatin. H2A is a part of the histone octomer made of pairs of histones H2A, H2B, H3 and H4 around which the DNA is wrapped. It is known that H2aV is expressed in early canoe stage and is gradually replaced by protamines in the late canoe stage (Rathke, Baarends et al. 2007). It is during this transitional phase in late canoe stage when CG5538 expression starts and for a transient period both proteins are present showing an overlay of red and green signals. In the next stage, the individualizing stage, the majority of histones are replaced with protamine resulting in compact nuclei. At this point the H2Av signal diminishes and only the green CG5538 signal is present. Once individualization is completed sperm nuclei take on their final shape resembling thin needles. In these mature clusters almost all histones have been replaced with protamines and CG5538 is no longer detectable (Figure 11).

**Figure 11. Expression Pattern of CG5538 and H2Av in Testes.** H2Av (red in overlay) is expressed post meiosis and is present from leaf stage peaking at mid canoe stage. H2Av is not present in the late canoe and mature needle stage as it is replaced by protamines. CG5538 expression (green in overlay) starts at early canoe stage, accumulates the greatest amount during mid-canoe stage and declines during late canoe stage. CG5538 is not present in mature sperm at needle stage.



## CHAPTER IV

### DISCUSSION

#### ***mcl(3)Z2566* is a Mutation in *CG5538***

The mutation *mcl(3)Z2566* was originally identified by Wakimoto et al. (Wakimoto, Lindsley et al. 2004) in a screen for mutations that increased the frequency of paternal fourth chromosome loss among offspring of mutant males. Here we present conclusive evidence that the gene disrupted by this mutation is *CG5538*.

First, we generated new *CG5538* alleles by P element transposition, *P{EP}CG5538<sup>G19873m1</sup>* and *P{EP}CG5538<sup>G19873m2</sup>*. Sequencing of one of these, *P{EP}CG5538<sup>G19873m2</sup>*, revealed a missense mutation in *CG5538* (G607A) resulting a change of Valine to Methionine in the conceptually translated amino acid sequence V(195)M. This new mutation had a phenotype similar to that of the original *mcl(3)Z2566* mutation, consistent with both mutations being loss-of-function alleles of *CG5538*.

Secondly, we showed that germline knock-down of *CG5538* expression by siRNA results in the same mutant phenotype of 4<sup>th</sup> chromosome loss. The frequency of chromosome loss was lower (~2%) compared to that seen from the *mcl(3)Z2566* mutant males, likely indicating an incomplete inhibition by RNAi. Nonetheless, inhibition of *CG5538* induced paternal chromosome loss whereas inhibition of *desat1* did not, supporting our conclusion that *CG5538* was the gene responsible for the *mcl(3)Z2566* phenotype.

Thirdly, we created transgenic flies bearing an EGFP-labeled copy of the wildtype *CG5538* gene and tested for rescue of the *mcl(3)Z2566* mutant. We showed that a single copy of the transgene restored the mutant to wildtype phenotype, reducing rates of sex and fourth chromosome loss to background levels.

### ***CG5538* Encodes a Novel Protein Unique to Diptera**

*CG5538* is a small gene of 1394 bp with one intron coding for a small basic protein (PI ~ 9.49) with 387 amino acids specifically expressed in testes (flybase.org). The protein does not have any sequence homology to other known proteins, thus identifies a new paternal function important for embryonic chromosome transmission.

Clustal W amino acid sequence alignment shows that *CG5538* is evolutionarily conserved between all *Drosophila* species and some other Dipterans (Figure 12), and it seems that the protein evolved before the split of *Drosophila* from other Diptera. The region of highest conservation between homologs is at the amino terminus, and the second amino acid, aspartic acid (D), is conserved in all *Drosophila*. The *mcl(3)Z2566* mutation replaces this residue with valine (V). The amino terminus of the protein is universally conserved among all Sophophora and *Drosophila* species suggesting that this domain is essential for function.

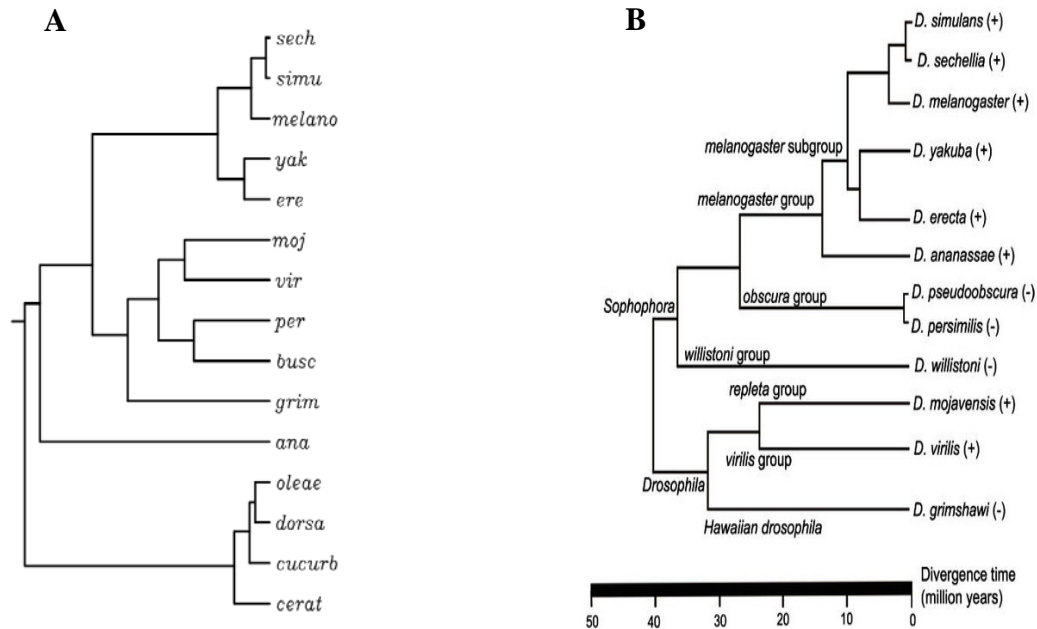
No mammalian homolog of *CG5538* could be identified. This may reflect an insect-specific requirement for male germline chromatin, or may mean that a different protein carries out a homologous function in mammals. Additionally we found no obvious relationship to other known genes in *Drosophila*, thus it identifies a new function important for chromosome transmission.

**Figure 12. ClustalW Alignment CG5538 Homologs in *Drosophila* Species and Related Diptera.**

<i>Drosophila melanogaster</i>	<b>MDEF</b> RV <b>P</b> KKV <b>NR</b> NV <b>F</b> KAISIL
<i>Drosophila simulans</i>	<b>MDEF</b> RV <b>P</b> KKV <b>NR</b> NV <b>F</b> KAISIL
<i>Drosophila secchelia</i>	<b>MDEF</b> RV <b>P</b> KKV <b>NR</b> NV <b>F</b> KAISIL
<i>Drosophila erecta</i>	<b>MDDEF</b> RV <b>P</b> KKV <b>NR</b> NV <b>F</b> KAISIL
<i>Drosophila yakuba</i>	<b>MDEF</b> RV <b>P</b> KKV <b>NR</b> NV <b>F</b> KAISIL
<i>Drosophila ananassae</i>	<b>MDLF</b> NV <b>P</b> KKV <b>NR</b> HV <b>V</b> KA <b>V</b> CFL
<i>Drosophila persimilis</i>	<b>MDNF</b> QV <b>P</b> KVV <b>NR</b> HVL <b>Q</b> AIYYF
<i>Drosophila mojavenis</i>	<b>MDNF</b> HV <b>P</b> KK <b>I</b> NRNV <b>L</b> KALGIL
<i>Drosophila grimshawi</i>	<b>MDQF</b> NV <b>P</b> RN <b>I</b> NRQ <b>V</b> FNA <b>V</b> VKL
<i>Drosophila virilis</i>	<b>MDQF</b> NV <b>P</b> KKV <b>NR</b> HV <b>F</b> KA <b>V</b> SAL
<i>Drosophila busckii</i>	<b>MDNF</b> DV <b>P</b> RKV <b>NR</b> HV <b>L</b> KA <b>I</b> CSI
<i>Bactrocera dorsalis</i>	<b>MDHF</b> NV <b>P</b> KKV <b>NR</b> HV <b>L</b> KALGVL
<i>Bactrocera cucurbitae</i>	<b>MDHF</b> NV <b>P</b> KKV <b>NR</b> HV <b>L</b> KALGVL
<i>Bactrocera oleae</i>	<b>MDHF</b> NV <b>P</b> KKV <b>NR</b> HV <b>L</b> KALGFL
<i>Ceratitis capitata</i>	<b>MDLF</b> NI <b>P</b> KKV <b>NR</b> HV <b>L</b> KALCAL
Conserved amino acids	<b>MDx</b> <b>F</b> x <b>V</b> <b>P</b> Kx <b>V</b> <b>N</b> Rxx <b>B</b> Z <b>A</b> Bxxx I R I



**Figure 13. Phylogenetic Tree of CG5538 and Sophophora Phylum.** A. Phylogenetic tree of the CG5538 protein within all *Drosophila* species. B. Sophophora phylum showing the relationship of all *Drosophila* species based on their genomes.



## Expression and Localization of CG5538

We took advantage of the transgene-produced CG5538-GFP fusion protein to examine the expression and localization pattern of CG5538 in the male germ line. We found that the GFP signal was undetectable until the early canoe stage, was most intense during the mid and late canoe stages of spermiogenesis, and was again undetectable at the individualization stage. Thus, we conclude that the expression of CG5538 is limited to the canoe stage. The protein was nuclear localized in a pattern that appeared to be chromatin-associated. This expression pattern is significant as this is the developmental stage at which histones are replaced by protamines. We did not detect *EGFP* signal at any premeiotic or meiotic stages – making it unlikely that the defect leading to chromosome

loss affects gonial divisions or meiosis. This leads us to suggest that the induced chromosome loss occurs in the embryo, and may be related to a defect in sperm chromatin remodeling.

We verified expression at the histone-to-protamine transition by examining CG5538-GFP in flies co-expressing H2Av-RFP. H2Av, like other core histones, is removed during the mid-canoe stage when protamines and Mst77F start accumulating in the nucleus (Rathke, Barckmann et al. 2010). The expression pattern of CG5538-EGFP mimics that reported for the transition protein-like proteins Tpl 94D, tHMG1 and tHMG2 (Rathke, Baarends et al. 2007). Although the exact function of CG5538 is yet to be determined, the similarity of the expression pattern and localization of CG5538 to the nucleus suggests the possibility of CG5538 being a novel member of this transition complex.

This role would be consistent with our observations on chromosome loss. We found that males mutant for *CG5538* showed increased sex and fourth chromosome loss among progeny, however we found no increase in diplo-exceptional progeny that received two copies of a paternal chromosome. Thus, it is unlikely the chromosome loss results from meiotic nondisjunction. Additionally, we found no cytological evidence for meiotic loss in examination of onion stage spermatids. Finally, the occurrence of mosaics suggests that at least some of the loss occurs in the embryo. The most parsimonious interpretation, consistent with the post-meiotic expression pattern of our transgene, is that chromosome loss is embryonic. The observation that all of these mosaics showed chromosome loss in roughly half of the tissues indicates that the loss in these flies

occurred at the first division. This may suggest that the defect in *mcl(3)Z2566* sperm is ameliorated after the first division. This might occur by chromatin remodeling in the embryo during the first division, when the replacement of paternal protamines by maternal histones occurs.

In our tests, *CG5538* mutants exhibited relatively low frequencies of sex chromosomes loss compared to 4<sup>th</sup> chromosome loss, and no detectable loss of the major autosomes. Similarly, the incidences of 4<sup>th</sup> and sex chromosomes losses induced by mutations in *pal* differ, at 17% and 3-4% respectively (Baker 1975). Unlike mutations in *CG5538*, however, *pal* also induces loss of the major autosomes. Mutations in *Horka* also show a chromosome-specificity, but it differs from that of *CG5538*. Males mutant for *Horka* exhibit loss of all chromosomes except the Y chromosome (Szabad, Mathe et al. 1995).

The significance of the *CG5538* chromosome specificity is unclear. It may reflect a size-dependent sensitivity as the 4<sup>th</sup> chromosome is much smaller in size compared to the sex chromosomes and the other major autosomes. It is possible that there is competition for a rate limiting factor in sperm chromatin assembly among chromosomes where chromosome 4 is disadvantaged due to its small size. Alternatively, larger chromosomes like the sex chromosomes and major autosomes that are lost at very low frequencies might be less prone to loss because more centromeric heterochromatin stabilizes some aspect of chromosome transmission, such as centromere function.

The other paternal-effect mutations also cause higher overall rates of chromosome loss compared to null *CG5538* mutations. The low frequencies of chromosome loss

suggest that the function of *CG5538* may be partially redundant with that of another gene. It is unlikely that the low levels of loss or chromosome specificity are due to the nature of the *mcl(3)Z2566* mutation. Genetic crosses with *P{EP}<sup>CG5538G19873m2</sup>*, allele with a point mutation at a different location in the gene showed similar chromosome loss phenotype which suggests that chromosome loss is not dependent on specific nature of *mcl(3)Z 2566* mutation, but reflects the loss-of-function phenotype for *CG5538*. The frequency of loss is also comparable in mutant/Df trans-heterozygotes.

### **Possible Functions of CG5538**

*CG5538* is small in size, is basic and is expressed only in testes, features shared with transition protein-like proteins. Like Tpl 94D, it is serine- and arginine-rich, but lacks a DNA binding HMG box domain typical of transition protein-like proteins. Since *CG5538* is expressed during the canoe stage as are transition protein-like proteins we hypothesize that *CG5538* is part of the transition complex during histone to protamine transition.

Alternatively, *CG5538* could also be part of a complex that is involved with incorporating CID, the centromere specific H3 variant retained in mature sperm, into the centromere region to replace H3 during canoe stage. Since CID is essential for centromere function of the paternal chromosomes in early embryogenesis and maternal CID cannot replace paternal CID in deficient chromosomes, lack of CID would cause loss of paternal chromosomes. Therefore we can speculate that *CG5538* mutants might indirectly lead to CID-deficient paternal chromosomes and ultimately to paternal

chromosome loss by undermining CID loading during canoe stage. This might be tested by examining CID localization in *mcl(3)Z2566* sperm.

Future experiments with GFP-labeled components of the transition complex (Tpl94D, tHMG1 and tHMG2) could show co-localization of CG5538 with these factors to the chromatin. Interaction assays with the same factors could test whether CG5538 is required for localization and whether its removal would interfere with the histone-to-protamine transition. Experiments with GFP-labeled histones and protamines (Mst35A, Mst35B) could reveal if histones are retained and if protamines are altered in the mutant. Another set of experiments would be to test epistasis with *pal*, *loser* and *ms(3)K81* to see whether any of these genes suppress the effect of *CG5538* or if the effect of any of these genes depends on the presence of *CG5538*. Antibody staining to epigenetic marks present during spermiogenesis in mutant could show if *CG5538* interferes with epigenetic transmission of these marks. Lastly, early embryos of mutant fathers should be observed directly by confocal microscope imaging to gain insight into the mechanism of chromosome loss in the embryo.

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